Bangladesh J. Plant Taxon. **27**(1): 173–184, 2020 (June)

EVALUATION OF GENETIC DIVERSITY AND MORPHOLOGICAL VARIABILITY IN *STELLARIA MEDIA* (L.) VILL. USING RAPD MARKER

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Keywords: Gene flow; Morphology; RAPD; Species relationship**;** *Stellaria media*.

Abstract

Stellaria media (L.) Vill., known under the name of chickweed, is an annual medicinal plant in the family Caryophyllaceae. This species is distributed in the north regions of Iran. In the present study, the Random Amplified Polymorphic DNA (RAPD) technique was used to estimate infraspecific variation in different populations of *Stellaria media*. Samples from 11 populations were collected from different regions of the country during spring 2018. For RAPD investigations, populations were divided into three geographical regions. This study provides important data on the genetic diversity, population structure and morphological characteristics of *Stellaria media*. The AMOVA and Gst analyses showed that the populations of this species are genetically differentiated. Nm analysis revealed very low value of genetic diversity among the studied population and mantel test indicated isolation by distance occurred among them. The studied populations of *S. media* are differentiated in morphological characteristics and genetic content.

Introduction

Genetic diversity, one of the basic levels of biodiversity, determines the evolutionary potential of a taxon or population to adapt to variable environmental conditions (Morton, 2005). Genetic diversity arises from different factors such as mutation or gene flow, while events, for example, genetic drift and directional selection, can decrease the diversity. The family Caryophyllaceae comprised of about 81 genera and 2600 species (Bittrich, 1993; Ullah *et al.*, 2019; Esfandani–Bozchaloyi *et al.*, 2017a,b,c,d; 2018a,b,c; Esfandani -Bozchaloyi and Sheidai 2018). *Stellaria* L. (Caryophyllaceae, Alsinoideae) includes both annual and perennial herbaceous plants that are widely distributed in the temperate zones of Europe and Asia (Lu and Rabeler, 2001; Keshavarzi and Esfandani–Bozchaloyi, 2014a, b; Ullah *et al.*, 2019) and about 120 species with worldwide distribution, mainly in the north temperate zone (Morton, 2005; Ullah *et al*., 2018). In Flora Iranica, this genus has nine species under two sections: sect. *Pseudalsine* Boiss. consists of one species *S. alsinoides* Boiss. & Buhse and sect. *Stellaria* of six species *viz*., *S. holostea* L., *S. persica* Boiss., *S. graminea* L., *S. nemorum* L., *S. media* (L.) Vill., and *S. pallida* (Dumort.) Pire (Rechinger, 1988). The main center of diversification for *Stellaria* is Eurasia, with a center of distribution in the mountains of central Asia. Some species are also cosmopolitan (Bittrich, 1993; Ullah *et al*., 2018).

There are limited chromosome records for *Stellaria* in the world. Basic chromosome numbers of x=10, 11, 12 and 13 have been reported for the genus (Federov, 1969 1974; Moore, 1973; Goldblatt, 1981). *Stellaria media*, known as chickweed, are annual and with slender stems, they have hairs on one side of the stem. The leaves are linear or oval, smooth or minutely, 13 to 17 ×

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1.5 to 7 mm. Flowers are hermaphrodite. Sepals prominently 4 to 6-nerved. The number of stigmas and stamens are 3 each. *S. media* is common in waste places, open areas, lawns, meadows, and widely distributed to temperate regions of Europe, Asia and Northern America. *S. pallida* is distributed in the all regions of Iran. This plant is edible and nutritious and considered to be a herbal remedy and useful in folk medicine. *S. pallida* is very similar to *S. media. Stellaria media* has some medicinal properties. This species has been used as to soothe severe itchiness even where all other remedies have failed (Slavokhotova *et al*., 2011). It is considered for rheumatic pains, skin diseases, and period pain as well as for bronchitis and arthritis (Slavokhotova *et al*., 2011). Rani *et al*. (2012) have studied some stem and leaf anatomical features through the pharmacognostical study for quality control of *S. media*. Arora and Sharma (2012) did pharmacognostical and phytochemical studies of *S. media* and showed the presence of epidermis, palisade cells, trichomes and vascular bundles in leaf. *S. media* possesses significant chemicals known as saponins, which can cause poisoning in cattle (Haragan, 1991). Many studies have been done on taxonomy, pollen morphology, phylogeny, seed micromorphology, anatomy, trichome and cytology of *Stellaria* species (Esfandani-Bozchaloyi and Keshavarzi, 2014; Keshavarzi and Esfandani-Bozchaloyi, 2014 a, b; Ullah *et al*. 2018). However, genetic diversity of *Stellaria* species have been reported only in a few studies (Verkleij *et al*., 1980; Chinnappa and Morton, 1984), and outcrossing or inbreeding, genetic structure, genetic variability within/between populations and ecological adaptation in *Stellaria* of Iran have not been investigated yet.

The molecular markers are extensively used in germplasm characterization, fingerprinting, genetic analysis, linkage mapping, and molecular breeding. RAPD (Random Amplified Polymorphic DNA) analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals. The advantages of this technique are: a) a large number of samples can be quickly and economically analyzed using only micro-quantities of material; b) the DNA amplicons are independent from the ontogenetic expression; and c) many genomic regions can be sampled with a potentially unlimited number of markers (Ellis and Burke, 2007; Esfandani-Bozchaloyi *et al.*, 2017a,b,c,d). The present investigation has been carried out to evaluate the genetic diversity and relationships among 11 geographical populations of *S. media* using RAPD markers. This is the first study on the use of RAPD markers in *S. media;* Therefore, we performed molecular study of 110 collected specimens of 11 geographical populations.

Materials and Methods

Morphological studies

110 plant sample were selected from eleven populations located in three provinces of Iran. Identification of *S. media* species were based on the descriptions provided by Flora Iranica (Rechinger, 1988). The sampling sites are provided in Table 1 and Fig. 1. Voucher specimens were deposited at the herbarium of Islamic Azad University, Science and Research Branch, Tehran, Iran (IAUH).

DNA extraction

Fresh leaves were used randomly from one to twelve plants in each of the studied populations. These were dried by silica gel powder. CTAB activated charcoal protocol was used to extract genomic DNA (Esfandani-Bozchaloyi *et al.,* 2018a,b,c). The quality of extracted DNA was examined by running on 0.8% agarose gel. 25 decamer RAPD primers of Operon technology (Alameda, Canada) belonging to OPA, OPB, OPC, OPD sets were used in this study. 10 primers with clear, enlarged, and rich polymorphism bands were chosen. PCR were carried out in 25 µl reactions containing 20 ng of template DNA, 0.3 mM dNTPs, 1μM primers, 1.0 μl of 20×PCR buffer (Cinnagen, Iran), 1.8 mM of $MgCl_2$ and 5 units of Taq polymerase (Cinnagen, Iran).

Table 1. Location addresses and ecological characters of the *Stellaria media*

Population	Locality	Latitude	Longitude	Altitude (m)	Voucher no.
1	Guilan, Road to Sangar	37° 06 ' 57"'	49˚ 11 ' 06 "	47	IAUH 201600
2	Guilan, Bandar Anzali, Pine artificial woodland	37 ˚27 '34 "	49˚ 42 ' 40 "	-25	IAUH 201701
3	Guilan, Loleman	37˚ 28 ' 59 "	49˚ 33 45 "	-29	IAUH 201702
4	Guilan, Siahkal, Sangar	37˚ 09 ' 08 "	49˚ 55 ' 02	27	IAUH 201603
5	Golestan, Golerodbar river	37˚ 10 ' 05 "	49˚ 56 ' 38 "	15	IAUH 201604
6	Guilan , Sheytankouh hill side	37˚ 12 ' 04 "	50˚ 03 ' 12 "	9	IAUH 201605
7	Guilan , Lahijan , Highlands of Sheytan Kouh	37˚ 11 ' 52 "	50˚ 03 ' 17 "	159	IAUH 201606
8	Guilan, Bandar Anzali, Road side	37˚27 ' 48"	49˚22 ' 30 "	-11	IAUH 201707
9	Mazandaran, ChalosNeamatabad	36˚ 49 ' 02 "	50˚ 52 ' 20 "	-16	IAUH 201608
10	Mazandaran, Shirodi Ring Road	36˚ 51 ' 10 "	50˚ 32 ' 11 "	-18	IAUH 201709
11	Mazandaran, Noshahr	36˚ 35 ' 04 "	51˚ 35 ' 14 "	-20	IAUH 201710

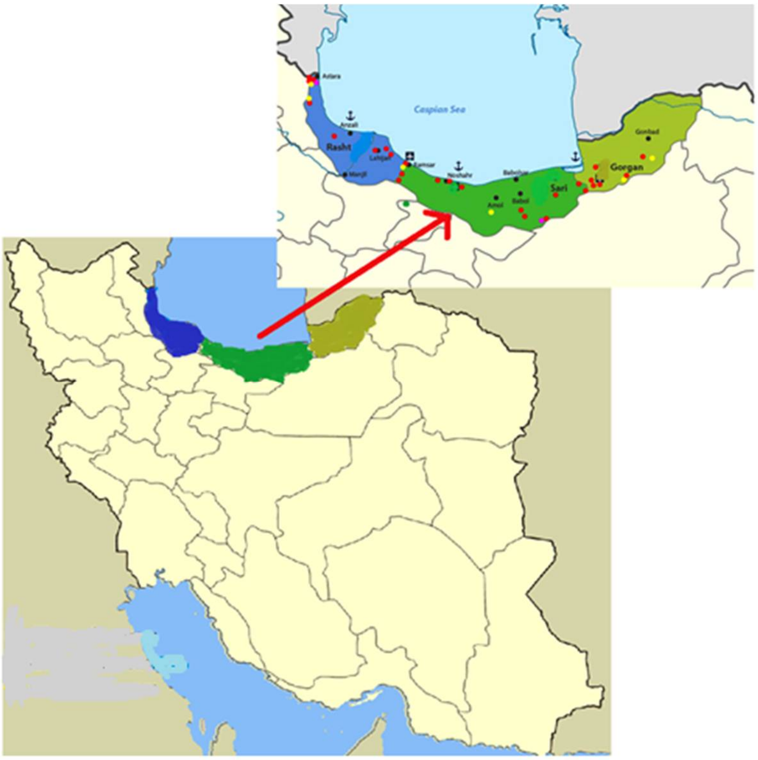

Fig. 1. Distribution map of the studied populations.

The amplification was carried out, with programmed as initial pre-denaturation at 95°C for 5 min followed by 36 cycles of denaturation at 94°C for 45 s, annealing at temperature (52-55°C) for 40 s, and extension at 72°C for 1min. A final 5 min. extension at 72°C followed the completion of 38 cycles.

Data analyses

Morphological studies

For morphological studies, 43 morphological characters including 16 qualitative and 26 quantitative characters were studied following Attar *et al.* (2019, Table 2).

Table 2. List of selected characters and their codes in morphological studies.

No.	Characters	Numerical code
1	Plant height	mm
2	Length of basal leaves	mm
3	Width of basal leaves	mm
4	Length of stem leaves	mm
5	Width of stem leaves	mm
6	Bract length	mm
7	Width bract	mm
8	Length pedicel	mm
9	Number of seeds per capsule	
10	Number of flowers per inflorescence	
11	Number of calyx	
12	Length calyx	mm
13	Width calyx	mm
14	Number of petal	
15	petal length	mm
16	Petal width	mm
17	Cleft size of petals	mm
18	Inter node length	mm
19	Number of stamen	
20	Number of stigma	
21	Capsule length	mm
22	Seed length	mm
23	Seed width	mm
24	Cleft size of capsule	mm
25	Number suture capsules	
26	Veins number sepals	

27	Growth period	0-annual 1- perennial
28	Bract apex	0-acute 1- narrow 2- absence
29	State of stem	0-unbranched 1- branched
30	State of stem strength	0-thin 1- strong
31	Hairs of stem	1-unilateral hair 2- multilateral hair
32	Cross-section of stem	0-round1- rectangular 2- elliptical
33	Shape of basal leaves	0-linear 1- linear- lanceolate
34	Basal leaves apex	0-acute 1- narrow
35	Basal leaves petiole	0-absence 1- presence
36	Hair of basal leaves petiole	0-absence 1- presence
37	Shape caulin leaves	0- linear 1- linear- lanceolate
38	Caulin leaves apex	0- acute 1- narrow
39	Caulin leaves petiole	0-absence 1- presence
40	Hair of caulin leaves petiole	0-absence 1- presence
41	Hair of caulin leaves margin	0-absence 1- presence
42	Hair of caulin leaves lamina	0-absence 1- presence
43	Shape of bract	0-linear 1- linear- lanceolate

Morphological traits were standardized (Mean = 0, Variance = 1) and used to estimate Euclidean distance for ordination analyses (Podani, 2000). PCA (Principal Components Analysis) biplot and MDS (Multidimensional Scaling) were applied for grouping and identifying the most variable morphological traits among the populations (Podani, 2000). We used PAST version 2.17 (Hammer *et al.*, 2012) for multivariate statistical analyses.

Molecular analyses

RAPD bands scored as present (1) or absent (0). Genetic polymorphism was determined by genetic diversity parameters: Shannon information index (I), percentage of polymorphism, the number of effective alleles and Nei's gene diversity (H) (Freeland *et al*., 2011). Neighbor-Net networking was used for Nei's genetic identity among studied populations (Huson and Bryant, 2006; Weising *et al*., 2005). We used PAST ver. 2.17 (Hammer *et al*., 2012), SplitsTree4 V4.13.1 ,2013 and DARwin ver. 5, 2012 softwares for data analysis.

For AMOVA (Analysis of molecular variance), we used GenAlex 6.4 software (Peakall and Smouse 2006; Meirmans and Van Tienderen, 2004) to determine the genetic differentiation of the species and Nei'sGst analysis in GenoDive ver.2 (2013) (Hedrick, 2005; Jost, 2008) were used to reveal genetic distance of the species. First data were scored as dominant markers so we used from STRUCTURE analysis for estimation of the parameters that is related to gene flow among studied population. Burn-in = 10000, and 10 runs were performed for estimation of the relationship between the genetic structures and geographical distance. Maximum likelihood method and Bayesian Information Criterion (BIC) was studied by structure analysis (Falush and Stephens 2007; Evanno *et al*., 2005; Meirmans, 2012). Gene flow was determined by calculating Nm from Gst by PopGene ver. 1.32, 1997 (Pritchard *et al*., 2000).

RESULTS AND DISCUSSION

In this study 11 populations of *Stellaria media* were selected from northern regions of Iran. Genetic diversity parameters revealed that the highest percent of genetic polymorphism (70%) and gene diversity (0.203) exist in Mazandaran, Chalos Neamat abad (population No.9), while the lowest amount of genetic polymorphism (22%) showed in population in Mazandaran, Noshahr (Table 3).

Table 3. Genetic diversity parameters in the studied populations. (N = number of samples, Ne = number of effective alleles, I= Shannon's information index, He = gene diversity, UHe = unbiased gene diversity, P%= percentage of polymorphism, populations).

Pop	N	Na	Ne	I	He	UHe	%P
pop1	5.000	0.839	1.134	0.128	0.083	0.092	25.81%
pop2	5.000	1.258	1.279	0.248	0.164	0.182	48.39%
pop3	5.000	1.097	1.220	0.214	0.137	0.153	45.16%
pop4	5.000	1.516	1.406	0.355	0.236	0.263	67.74%
pop5	5.000	1.323	1.230	0.220	0.143	0.158	45.16%
pop6	5.000	1.516	1.411	0.353	0.238	0.264	64.52%
pop7	5.000	1.194	1.248	0.254	0.161	0.179	54.84%
pop8	5.000	1.226	1.258	0.257	0.162	0.180	58.06%
pop9	5.000	1.452	1.318	0.320	0.203	0.226	70.97%
pop10	5.000	0.968	1.085	0.115	0.066	0.073	32.26%
pop11	5.000	0.645	1.092	0.101	0.063	0.070	22.58%

AMOVA test showed that, 35% of total genetic diversity was within population and 65% was among population. Hedrick standardized fixation index makes the genetic distance among the studied populations. We have moderate level for AMOVA produced after 999 permutations (G'st = 0.632, P = 0.001) and Hedrick differentiation index (D-est = 0.271, P = 0.001). Our results showed that the populations of *S. media* are differentiated from each other.

Populations' genetic affinity

Neighbor-Net network and Nj tree revealed identitical results but here only Neighbor-Net network is discussed (Fig. 2). This network shows that the populations 3 and 7, as well as populations 4 and 11 are placed close to each other, which indicate that they have closer genetic affinity. The populations 2 and 6 and 8 are differentiated from the other populations. The studied specimen in PCoA plot revealed that they belong to different groups, which is in agreement with the AMOVA results (Fig. 3). The relationship between altitude distance and genetic distance indicated by Mantel test after 5000 permutations is significant in these populations (r = 0.22, P = 0.001). The isolation in *S. media* occurred because of low amount of gene flow due to geographically more distant of populations.

Populations genetic structure

The result carried out on STRUCTURE analyses by Evanno test makes a peak at k = 6 (Fig. 4). Furthermore, STRUCTURE analyses show genetic identity between populations 3 and 4 (similarly colored), populations 5 and 6, like populations 10 and 11. But it indicated genetic difference between populations 1 and 2 (differently colored).

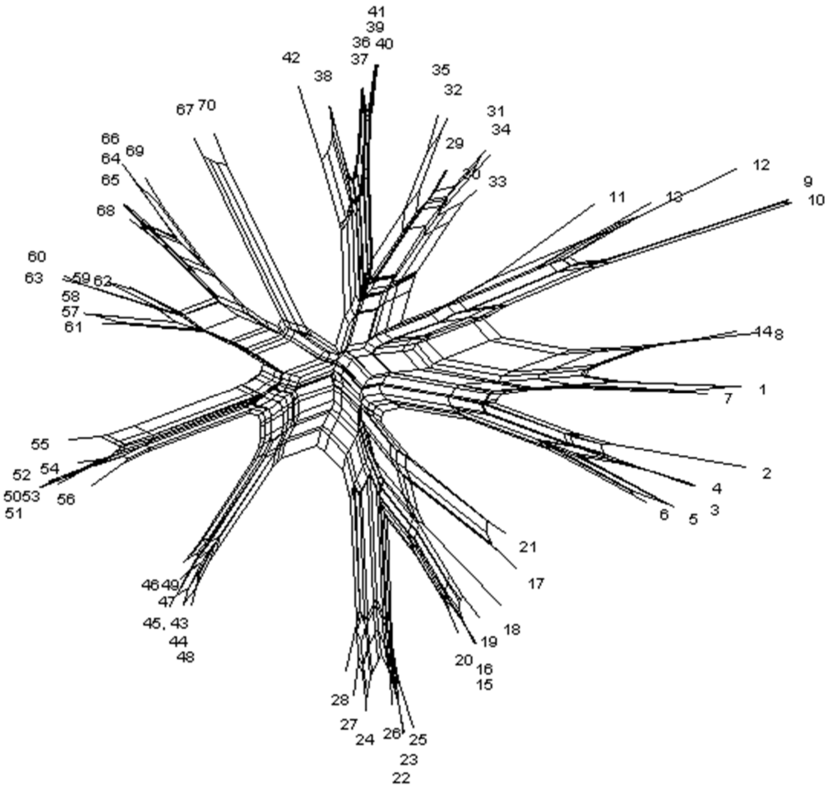

Fig. 2. Neighbor-Net network of populations in *S. media* based on RAPD data.

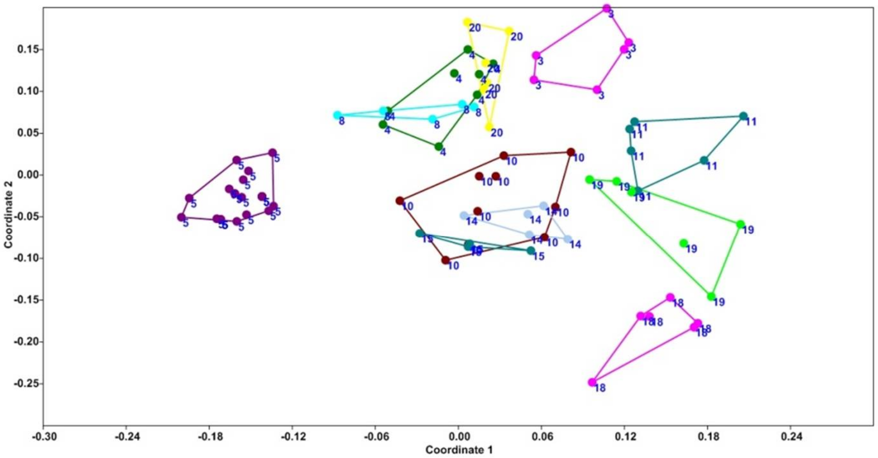

Fig. 3. PCoA plot of populations in *S. media* based on RAPD data.

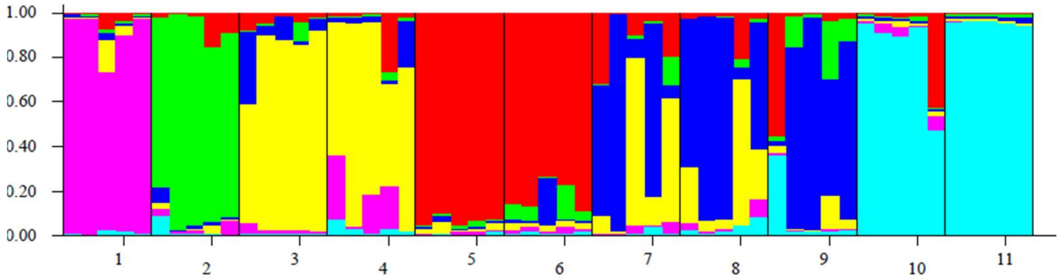

Fig. 4. STRUCTURE plot of *S. media* populations based on k = 6 of RAPD data.

The results of Reticulogram (Fig. 5), that is based on the least square method, indicates some of shared alleles among populations 3 and 5, 6 and between 8 and 2 and 11, also between 1, 4, 3 and 9 and 10. The mean Nm = 0.32 indicates very low level of genetic diversity and supports genetic stratification as showed by STRUCTURE analyses and K-Means. Nm result agreed with population assignment test and cannot showed gene flow among these populations. In total, ten ISSR primers produced 90 bands, fragment size ranged from 150 to 3000 bp.

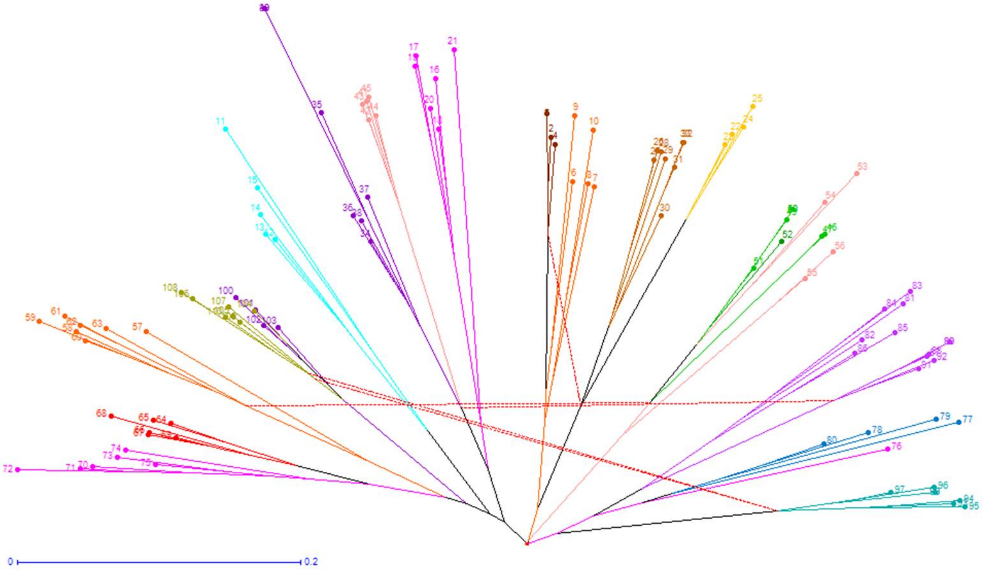

Fig. 5. Reticulogram of *S. media* populations based on least square method analysis of RAPD data (Population numbers are according to Table 1).

Morphometric analyses

ANOVA tests for 110 plant specimens from 11 populations were performed. Our results indicated significant difference in comparison with the studied populations ($P < 0.05$). Ordination plot and other analyses produced similar results on these populations (Fig. 6). Our result revealed that among of the studied populations, morphological divergence exist and this divergence was due to quantitative traits. For example, length of stem leaves character separated population No. 2, but the populations 4 and 8 separated from the other populations due to difference in calyx length. We performed for both morphological and RAPD data-based a consensus tree (Fig. 7). It indicates that some populations are different from other populations due to both morphological and molecular characters.

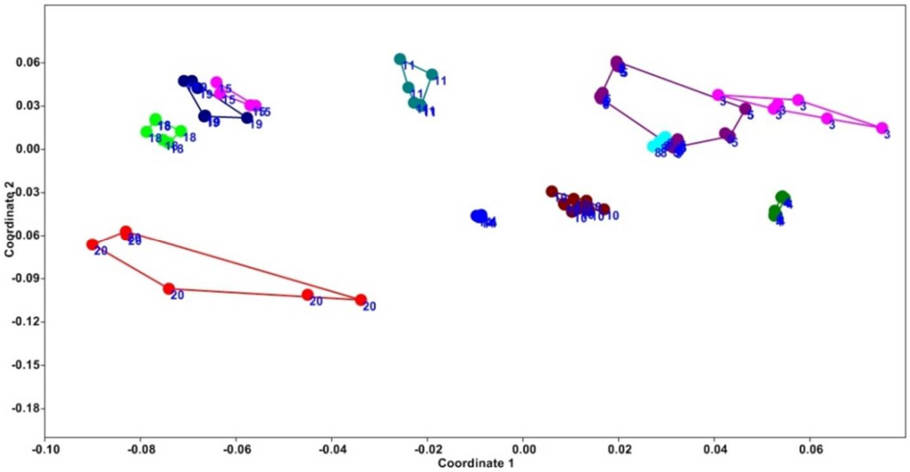

Fig. 6. PCoA plot of *S. media* populations based on morphological characters.

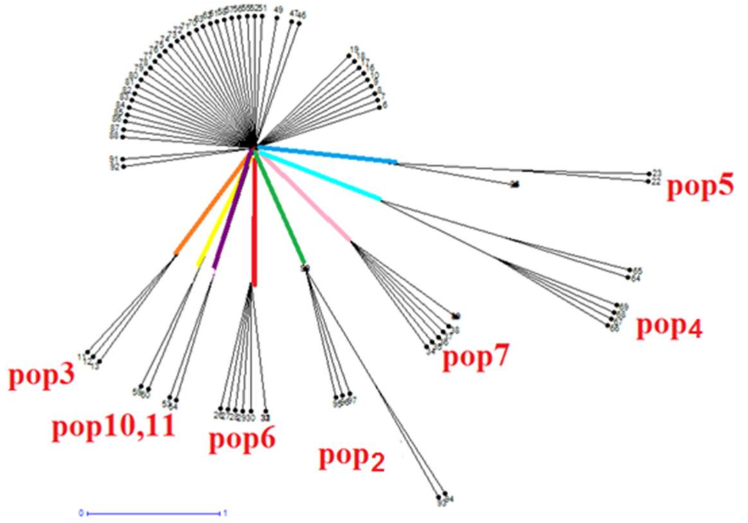

Fig. 7. Consensus tree of morphological and molecular data in *S. media* populations.

According to Çalişkan (2012) genetic diversity provides information about adaptation to changing environments, understanding of positive influence in the conservation of endangered species, hybridization and gene flow among the populations. This study evaluates the use of RAPD markers for comparing the gene flow and relationships within the population of *S. media* in Iran. Verkleij *et al*. (1980) showed that Amylases isoenzymes could be successfully applied to assess inter-populational variation in *S. media.*

In this study, we have provided information on current taxonomic, molecular and geographical distance and data about gene flow and genetic structure of *S. media* in some parts of Iran. Chickweed can germinate and flower any time of the year. It is mainly self-pollinating, but sometimes cross-pollination occurs by flies and insects.

According to Chater and Heywood (1993) *S,. media* is a widespread weedy species. There are three subspecies *viz*., *S. media* subsp. *media*, *S. media* subsp. *cupaniana* and *S. media* subsp. *postii* but some authors showed that subsp. *cupaniana* (Scholte, 1978) and subsp. *postii* (Sinha, 1965) should be included in *S. neglecta*. According to Fedorov (1969) chromosome numbers that have been reported for *S. media* included $2n$ = 24, 28, 36, 38, 40, 42 and 44 from many parts of the world. However, chromosome numbers $2n$ =40, 42 and 44 are the most commonly reported and this species shows a high degree of genotypic variation that is highly correlated with its reproductive characters (Freeland *et al*., 2011).

S. media is annual, characterized by the presence of five sepals and petals which are usually bifid. Generally, within family Caryophyllaceae diversity of morphological features makes taxa complicated to be delineated and identified. *S. media* is occur on abandoned fields and commonly sensitive to disturbance of its habitat. *S. pallida* and *S. media* are self-pollinating and there are crossing barrier between them. This happened due to the presence of polyploidy in *S. media* ($2n$ = 40-44), in contrast to the diploidy of *S. pallida* ($2n = 22$) (Scholte, 1978; Slatkin, 1993; Jolivet and Bernasconi, 2007). Therefore, breeding systems plays important role in low level of gene flow in *S. media* (Hutchison and Templeton, 1999; Medrano and Herrera, 2008).

Our results shows that the seed morphologies of *Stellaria media* and *S. pallida* are similar. Seed coat cellsare rounded polygonal and V-shaped margin. Based on these characters, we decided that *S. media* could be differenced from *S. pallida*. Seed coat morphology was observed in 18 species of *Stellaria* by Chen (2010).

Conflict of interests

The authors have not declared any conflict of interest.

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(Manuscript received on 24 January 2020; revised on 11 May 2020)